

Inactivation of PadR, the Repressor of the Phenolic Acid Stress Response, by Molecular Interaction with Usp1, a Universal Stress Protein from *Lactobacillus plantarum*, in *Escherichia coli*[∇]

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The phenolic acid decarboxylase gene *padA* is involved in the phenolic acid stress response (PASR) in gram-positive bacteria. In *Lactobacillus plantarum*, the *padR* gene encodes the negative transcriptional regulator of *padA* and is cotranscribed with a downstream gene, *usp1*, which encodes a putative universal stress protein (USP), Usp1, of unknown function. The *usp1* gene is overexpressed during the PASR. However, the role and the mechanism of action of the USPs are unknown in gram-positive bacteria. Therefore, to gain insights into the role of USPs in the PASR; (i) a *usp1* deletion mutant was constructed; (ii) the two genes *padR* and *usp1* were coexpressed with *padA* under its own promoter as a reporter gene in *Escherichia coli*; and (iii) molecular in vitro interactions between the PadR, Usp1, and the *padA* promoter were studied. Although the *usp1* mutant strain retained phenolic acid-dependent PAD activity, it displayed a greater sensitivity to strong acidic conditions compared to that of the wild-type strain. PadR cannot be inactivated directly by phenolic acid in *E. coli* recombinant cultures but is inactivated by Usp1 when the two proteins are coexpressed in *E. coli*. The PadR inactivation observed in recombinant *E. coli* cells was supported by electrophoretic mobility shift assays. Although Usp1 seems not to be absolutely required for the PASR, its capacity to inactivate PadR indicates that it could serve as an important mediator in acid stress response mechanisms through its capacity to interact with transcriptional regulators.

Phenolic acids are essential plant compounds involved in the molecular bonds between cellulose, hemicellulose, and lignin in the cell wall. They are significant in the mammalian diet (32). Their specific structure confers important biological activities. They have free radical scavenging properties that can trigger antimutagenetic effects (14) but that can also lead to pro-oxidant DNA degradation mediated by divalent cations (37). Recently, it was shown that some phenolic acids that induce the phenolic acid stress response (PASR) in gram-positive bacteria regulate the expression of genes of the type III secretion system required for virulence by the plant pathogen bacterium *Dickeya dadantii* (23). Phenolic acids such as *p*-coumaric, ferulic, and caffeic acids are toxic for numerous gram-positive bacteria such as *Bacillus subtilis* (33), *Pediococcus*

pentosaceus (4), and *Lactobacillus plantarum* (16) under acidic condition. Phenolic acid decarboxylase (PAD) activity in these bacteria is a detoxifying system specifically and strongly induced by these chemicals. Two genes involved in the PASR have been characterized: *padA* and *padR*. The *padA* gene (named *padC* in *B. subtilis*) encodes the PadA enzyme and *padR* encodes the PadR transcriptional repressor. Deletion of *padA* leads to growth inhibition in the presence of phenolic acids, especially at low pH (3), while deletion of *padR* leads to constitutive overexpression of *padA* (16) and, consequently, to high resistance to phenolic acids. Although the PadR-like proteins constitute a regulatory family of more than 1350 putative members in the bacterial genome database (accession no. Pfam PF03551), only a few of them are currently under investigation. Among them, the best characterized is AphA, the repressor of the penicillin amidase gene (21) and the transcriptional activator of the virulence cascade of *Vibrio cholerae*, which acts in consortium with the coactivator AphB (20). Another member of this family, LmrR, acts as a repressor of the multidrug recognition operon *lmrCD* in *Lactococcus lactis*, and its molecular structure in the presence or absence of H33342 or daunomycin drugs has been analyzed (26). As well as for the other PadR like proteins family, the way by which PadR is inactivated remains also poorly elucidated. Concerning the PASR, we have previously demonstrated that in growing cultures of a recombinant *Escherichia coli* strain expressing PadR from *P. pentosaceus* and in *L. plantarum*, PadR could not be inacti-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i>		
TG1	<i>supE hsdΔ5thi Δ(lac-proAB)F'</i> [<i>traD36 proAB+ lacI^a lacZΔM15</i>]	15
BL21(DE3) Star	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm</i> (DE3)	Invitrogen
TOP 10	F ⁻ <i>mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
<i>L. plantarum</i>		
NC8 (wild type)	Wild type, gram-positive ubiquitous homolactic acid bacterium, plasmid-free strain	2
LPNC8ΔUSP1 (Δ <i>usp1</i>)	<i>L. plantarum</i> NC8 strain with <i>usp1</i> disrupted by double homologous recombination	This study (Fig. 3)
LPNC8ΔPADR (Δ <i>padR</i>)	<i>L. plantarum</i> NC8 strain with <i>padR</i> disrupted by double homologous recombination	16; Fig. 3
Plasmids		
pTZ19R	Amp ^r ; Δ <i>lacZ</i>	Novagen
pET28a+	Kan ^r ; vector for overexpression of His-tagged proteins using the T7 bacteriophage promoter	Novagen
TOPO PCR 2.1	Amp ^r Kan ^r ; Δ <i>lacZ</i> , vector for TA cloning	Invitrogen
pGID023	Shuttle vector for <i>E. coli</i> and <i>L. plantarum</i> ; derivative of pJDC9 containing the pE194 replication functions; used as an unstable integration vector; Erm ^r	18
pJPDC1	Em ^r ; pJDC9 containing the 2.3-kbp <i>Sau3A</i> fragment of <i>L. plantarum</i> corresponding to the locus with <i>padA-padR</i> genes	6; Fig. 4
pLOCPAD	Amp ^r Kan ^r ; TOPO vector containing the 2.212-kbp LOCPAD fragment with <i>padA</i> , <i>padR</i> , and <i>usp1</i> PCR amplified with primers LPLOC1 and LPLOC2	This study (Fig. 4)
pTD14	Amp ^r ; pTZ19R containing the 311- and 385-bp DNA fragments PCR amplified with the primers LPDELU1-LPDELU2 and LPDELU3-LPDELU4, respectively	This study
pGΔUSP1	Em ^r ; pGID023 containing the 694-bp D14 fragment PCR amplified with the primers LPDELU1 and LPDELU4	This study
pED	pET28a+ with <i>padA</i> under its own promoter cloned into <i>SphI</i> restriction site	This study (Fig. 4)
pER	pET28a+ containing <i>padR</i> between <i>NcoI</i> and <i>XhoI</i> sites to produce PadR	16; Fig. 4
pEU	pET28a+ containing <i>usp1</i> between <i>NcoI</i> and <i>XhoI</i> sites to produce Usp1	This study (Fig. 4)
pEDR	pER with <i>padA</i> under its own promoter cloned into <i>SphI</i> restriction site	This study (Fig. 4)
pERU	pET28a+ with <i>padR</i> and <i>usp1</i> cloned between <i>NcoI</i> and <i>XhoI</i> restriction sites	This study (Fig. 4)
pEDRU	pERU with <i>padA</i> under its own promoter cloned into <i>SphI</i> restriction site	This study (Fig. 4)

^a Em^r, erythromycin resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

vated by exogenously added phenolic acids, even when the expression of *padR* was low (4, 16). We recently showed that partial inactivation of PadR was possible by the addition of phenolic acid at the onset of growth of a recombinant *E. coli* strain expressing the *padC* and *padR* genes from *B. subtilis* (33). However, this inactivation was very limited compared to that observed in phenolic acid-induced *B. subtilis* cultures. In *L. plantarum*, *padA* and *padR* form a divergon, and *padR* was predicted to be coexpressed with *usp1*, whose product displays significant identity with proteins belonging to the universal stress protein (USP) family (16). Expression of the putative bicistronic operon *padR-usp1* is induced by the addition of phenolic acids to the culture medium (24). Taken together, these data suggest that Usp1 might be a mediator of PASR by inactivating PadR, or a putative transcriptional activator of *padA*, which could enter in competition with PadR for its promoter. In order to study the role of *usp1* in PadR inactivation, we deleted the *usp1* gene from the wild-type *L. plantarum* strain or coexpressed it with *padR* and *padA* in *E. coli*, a species devoid of the PASR. Although the *usp1* deletion mutant still retained inducible PAD activity, we demonstrate that Usp1 inactivates PadR in recombinant *E. coli* strains. These results were confirmed by electrophoretic mobility shift assays (EM-SAs) with a *padA* promoter probe and PadR. We also demonstrate that Usp1 is involved in the acid stress response in cells entering into stationary growth phase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. *L. plantarum* LPNC8 strain, kindly given by Lars Axelsson (Matforsk, Oslo, Norway), was routinely grown in MRS agar or liquid medium (9) at 37°C without shaking, and *E. coli* was grown aerobically in Luria-Bertani (LB) medium at 37°C. The growth of bacteria was evaluated by monitoring the absorbance of culture at 600 nm with the appropriate dilution. Antibiotics were used in the following concentrations in the corresponding media: ampicillin, 200 μg/ml; erythromycin, 100 μg/ml; and kanamycin, 50 μg/ml for *E. coli* and 5 μg of erythromycin/ml for *L. plantarum*. To test bacterial resistance to acid shock, 100-μl samples of bacteria grown in MRS medium at an initial pH of 6.5 at 37°C were diluted 100-fold in a 10-ml volume of MRS medium with a pH 2.5. Cells were incubated for 1 h at 37°C. Viability was determined by CFU counts of culture dilutions plated onto MRS agar plates.

DNA manipulation, PCR amplification, and transformation procedures. DNA manipulation, purification, ligation, restriction analysis, and gel electrophoresis were carried out as described by Sambrook et al. (31). *L. plantarum* chromosomal DNA was prepared by the method described by Posno et al. (30). PCR was performed with 0.1 U of *Taq* DNA polymerase (MP Medicals), according to the manufacturer's recommendations, in an automatic thermocycler (Bio-Rad). Custom primers (Eurogentec) are listed in Table 2. PCR, and restriction products were purified by using a QIAquick PCR purification kit or a QIAgel agarose gel extraction kit (Qiagen). *E. coli* and *L. plantarum* strains were transformed by electroporation as described by Dower et al. (12) and Aukrust and Nes (1), respectively. *E. coli* BL21(DE3) Star was transformed by the CaCl₂ procedure (7).

Whole-RNA extraction. Cells from a 20-ml culture of *L. plantarum* were harvested and washed in cold ultrapure water by centrifugation and immediately resuspended in 1 ml of Tri-Reagent (Sigma) containing 100 mg of 50- to 70-μm glass beads. Total RNA was extracted immediately by disrupting the cells using a FastPrep System (MP Medicals). After this step, the procedure for total RNA

TABLE 2. Primers

Function and primer	Sequence (5'→3')	Site created ^a
Probe synthesis		
LPD1	ACCGACACTGATCCACTCAT	PstI
LPD2	CACCGATCTCGTCATCAAACG	
LPD3	CACCGATCTCGTCATCAAACG	
LPD4	GTCTAATATGTCGTTTTAAATC	
LPD5	GACTG <u>CAGG</u> CGACCGTTTCCCGC AAGC	
LPU1	AATGACGTCATCCACATCAC	
LPU2	TCAGAACGTGCGTTTTCGATA	
LPU3	GTCGAAGATCCAAGCTAAAC	
Deletion of <i>uspI</i>		
LPDELU1	GATAAGCTTTATAGCCATCCTTGCT	HindIII
LPDELU2	GTCTGCGAGTTCTAAGATTGCTGG	PstI
LPDELU3	TTGCTGCGAGGATAGACAAGTGGCT	PstI
LPDELU4	AAC <u>CGGATC</u> CAAGTTAGCACACTTAC	BamHI
Plasmid construction		
LPDLOC1	AATGAACAATAGCAGTCAAAACAA	
LPDLOC2	ACGTTTGTCTACCACCATTT	
LPD6	GGAGCATGCTAATGGTTGCTGGTTTA	SphI
LPD7	GGAGCATGCGAGTTTGTCTACCAC	SphI
LPU4	AAGCCATGGAATAACAAAAATGC	NcoI
LPU5	AATCTCGAGTTACCGAACAACGAT	XhoI
LPR1	CCCATGGCGCAAAAAATAAGTTACAA	NcoI

^a The sites are underlined in column 2.

purification was carried out according to the guidelines of the Tri-Reagent manufacturer. DNA traces were eliminated by incubating samples at 37°C for 40 min with 3 U of DNase I (MBI Fermentas). This enzyme was then inactivated for 10 min at 65°C. The effectiveness of the DNase treatment was checked by the absence of amplicons in PCR amplification of samples.

Southern and Northern blot analyses. The Southern blot was carried out with the *usp1* DNA probe on the *L. plantarum* genomic DNA digested with EcoRI. For Northern blotting, total RNA were resolved by denaturing formaldehyde agarose gel electrophoresis and transferred to nylon membranes (Nytran; Schleicher & Schuell) by using the Pharmacia vacuum system. *padR*- and *usp1*-specific probes were PCR amplified with the primer pairs LPD1/LPD2 and LPU1/LPU2, respectively, designed from *L. plantarum* NC8 chromosomal DNA. After purification, the DNA probe was labeled with [α -³²P]dATP (3,000 Ci/mmol; Perkin-Elmer) by random priming (Invitrogen). Transcript size was determined by comparison with an RNA ladder (0.24 to 9.5 kb; Invitrogen).

Plasmid constructs. All of the constructs used in the present study are presented in Fig. 3. Plasmid pLOCPAD was obtained by cloning into the vector TOPO TA (Invitrogen) the whole PCR-amplified (with the primers LPDLOC1 and LPDLOC2) 2.212-kbp *padA* DNA locus, including *padA*, *padR*, and *usp1* under their own promoters and transcriptional terminators. The pEDR plasmid was constructed by inserting the 979-bp *padA* fragment into the SphI restriction site of plasmid pER (16). The *padA* DNA fragment encompassing the first 60-bp of *padR* gene (16), the *padA* coding sequence, and the 267-bp downstream the TAA stop codon containing the transcriptional terminator was PCR amplified with the primers LPD6 and LPD7. Plasmids pEU and pERU were obtained by inserting the NcoI-XhoI DNA fragments corresponding, respectively, to the *usp1* coding sequence and the *padR* and the *usp1* coding sequences between the NcoI and XhoI sites of pET28a+. The DNA fragments were PCR amplified with the primers LPU4 and LPU5 for *usp1* and the primers LPR1 and LPR2 for the operon *padR-usp1*. The TAA stop codon of *usp1* was conserved to produce a native Usp1 protein without His tag. The plasmid pEDRU was derived from pERU by inserting the *padA* DNA fragment into the SphI restriction site (see the details above). The *padR* gene or the *padR-usp1* operon was cloned under the control of the T7 promoter of vector pET28a+, and resulting plasmids were transformed into *E. coli* BL21(DE3) Star (Invitrogen).

Knockout of *usp1*. The plasmid pTD14, which carries *usp1* with a deletion of a 352-bp fragment between 37 bp downstream of the start codon and 73 bp upstream of the stop codon, was constructed by simultaneously cloning two separate DNA fragments between HindIII and BamHI sites of pTZ19R. Primers LPDELU1 and LPDELU2, which include an HindIII and a PstI site, respectively, were used to amplify the 311-bp region encompassing the last 73 bp of *usp1*, and the 238-bp region downstream of *usp1*, which contains the transcrip-

tional terminator of the putative *padR-usp1* operon (Fig. 3A) (16). The 385-bp DNA fragment encompassing the 157-bp upstream of the stop codon of *padR*, the 190-bp region between the stop codon of *padR* and the ATG codon of *usp1*, and the first 37 bp of the 5' end of *usp1* were PCR amplified with the primers LPDELU3 and LPDELU4, which include PstI and BamHI restriction sites, respectively. The 575-bp fragment encompassing the two 311- and 385-bp fragments were PCR amplified from plasmid pTD14 with primers LPDELU1 and LPDELU4 and cloned between the HindIII and BamHI sites of the vector pGID023 to obtain plasmid pGΔUSP1 (Table 1). This plasmid was transformed into *L. plantarum* NC8, and transformants were selected on MRS medium containing 5 μ g of erythromycin and 12 μ g of lincomycin/ml. A few colonies were grown individually without antibiotics to produce 50 generations, and a diluted sample of each culture was poured onto MRS solid medium also without antibiotics. About 20 colonies were tested for simultaneous resistance to erythromycin and lincomycin. Five sensitive colonies were then screened by PCR amplification, with appropriate primers, to select clones giving the expected *usp1* gene deletion (Fig. 3A and B). Sequencing (Cogenics, France) of the amplicon obtained with the primers LPDELU1 and LPDELU4 was performed to confirm the deletion.

Cell extracts, assays for PAD activity, protein purification, and electrophoresis. *L. plantarum* and recombinant *E. coli* strain cultures, as well as cell disruptions, were performed as previously described (3) by using a Z Plus series cell disrupter (Constant System) (two passages at 10⁸ Pa). The PAD activity in the remaining whole cells and in cell extracts was measured as previously described (3), which consists of monitoring the disappearance of absorption peaks of the phenolic acid substrates and the simultaneous appearance of new peaks corresponding to the vinyl derivatives by UV spectrophotometry. The protein concentration in cell extracts was determined by using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard. For overexpression of PadR and Usp1 proteins, 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) was added to the appropriate cultures of *E. coli* at the mid-exponential growth phase, and incubation was continued overnight at 37°C. Cells were harvested by centrifugation (4,000 \times g), washed with saline, suspended in 25 mM phosphate buffer (pH 8) to 2% of the initial culture volume, and disrupted with the Z Plus series cell disrupter. His-tagged PadR protein was purified by overexpression and purification over a 0.5-ml nickel-nitrilotriacetate (Ni-NTA) agarose column (Qiagen). The PadR protein was eluted with an imidazole gradient of 20 to 100 mM. Protein extracts were resolved by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% polyacrylamide resolving gel, as previously described (16). To study the interaction between PadR and Usp1, extracts from recombinant *E. coli* expressing separately or simultaneously the two proteins were mixed together and resolved in 10% (wt/vol) PAGE (60 V, 10 h) without SDS denaturation or heating.

EMSAs. The 189-bp DNA probe corresponding to the promoter region of *padA* was PCR amplified with primers LPD3 and LPD4 and labeled with T4 polynucleotide kinase (Invitrogen) in the presence of [γ -³²P]ATP (Perkin-Elmer). Standard EMSAs were performed as follows. Purified PadR (from 2 to 20 nM) was incubated for 20 min at 28°C in 15 μ l of binding buffer containing 10 mM Tris-HCl (pH 7.8), 5% (vol/vol) glycerol, 0.2 mM EDTA, 50 mM KCl, 2 mM MgCl₂, 2 mM dithiothreitol, 2.5 μ g of bovine serum albumin/ml as unspecific protein competitor, and 2.5 μ g of salmon sperm DNA/ml as an unspecific DNA competitor. To verify the specificity of the binding of PadR to the *padA* promoter, 50- and 200-fold excesses of specific unlabeled *padA* promoter fragment or a 1,000-fold excess of unspecific competing poly(dI-dC) was tested as previously described (33). The samples were resolved onto 5% (wt/vol) PAGE gels, which were dried and analyzed by autoradiography. For testing the effect of phenolic acids on the binding, PadR was first incubated with different concentrations of *p*-coumaric acid in 15 μ l of binding buffer at room temperature for 15 min. The probe was then added in the above mixture and incubated for 20 min at 28°C before loading in a 5% polyacrylamide gel. For testing the effect of Usp1 on PadR, the protein extract from the Usp1 overexpressing *E. coli* pEU (U) strain, where Usp1 represents ca. 90% of the total proteins, was used. An extract of the *E. coli* strain with the vector pET28a+ without *usp1* was used as a negative control.

Glutaraldehyde cross-linking of PadR and Usp1. A modified procedure of Derré et al. (10), described by Gury et al. (16), was used for the cross-linking of PadR and Usp1. Glutaraldehyde was used at concentrations ranging from 0.25 to 1% (vol/vol). The samples were heated with loading buffer and analyzed by SDS-PAGE on a 12% (wt/vol) gel. The gel was stained with Coomassie blue.

LPUSP1/LP_3663	MENQKMQE----PLVYRRILLTVDEDDNTSSERAFRYATTLAHDYDV-PLGICSVLESEDINIFDLSL-TPSKIQAQRKHVEDVVAEYVQL	84
LVIS_1926	MADESDS ----PLVFRILLITIDEDDRPSTSRARFALTIAHDYQA-HLGIASVLESNDISIYESL-MPAKLNKREHLKTIYQYAAK	83
LSEI_2147	MVTEDDLTIE--SMHYNRILLAVDDDDSSSRKALNYACTVAHYDI-PLGIVSVLETGDLNIFQSL-TPDDYVHGQRSDLQQLHLSYQQL	86
LSA0042	MTEKQPLNIEVTSMHYQKLLIAIDDDPTSSKRAFNYACTVAKMYQI-PMGIVSILETSDLNIFYQSL-SPNVVAKRREEIAADLDIFVQK	88
PPYP_803599	MTFDVKRILVGVGD--DSEDVLAFLDYAINLAKKSGS-ELVIVSVLESDEMNVFQAL-DKDFIHGERAELEQHILKYQKK	75
LVIS0223	MTEK-----KIFTKILVGVGD--DSADQLAFKYAIKRARLEDA-ELYIVSVLESDDRSVYEAL-SKAFIHGERAQLVEHVNEYVQQ	77
LVIS_1577	MDNMKSMN----PKQVKRILVGVGD--DSPDAQLAFRYAMNRAKTDQA-ELVICSVLESNDNMNIYQAL-SKDYVHGQRSDLQQLHLSYQQL	82
LP_2877	MSEMS-----PKEFKHILVGVGD--DSDDAQLAFRYAINRAKSDGA-KLTIVSILEQDNMNVYEAM-SKDFVHGQRKDLLEHVQYQYKL	79
PPYP_803813	MSSREFN-----PKRFSKILVGVGD--DSPDAQLAFRYAMNRAKQDSS-ELDLVTVEIENDINIYQAL-DKNFITQKRTELEKHLQNYRKI	81
OENOO_6501	MSEKPI-----DKVFNRILVGVGD--DSADAQLAFRYAVRRAVKDNA-TLIITSILENDEMNVYQAL-TKDYVHGERDELVEHLKYYRDI	80
LP_2993	MATKFNRLVGVGD--DSADALAFDYAIHQAKQDGA-ELVIISILENDEMNVYQAL-SKDYIHGERQELQHLKYYQKQ	75
MJ0577	MSVMYKKILYPTDFSETAEIALKHVKAFKTLKAEVILLHVIDEREIKKRDIFSLLLGVAGLNKSVVEEFENELKNKLTE	79
ATP contacts:	<div style="display: inline-block; width: 100px; text-align: center;">A</div> <div style="display: inline-block; width: 100px; text-align: center;">A</div>	
Consensus%.iL..vD..#.laf.yA...ak.....L!.svlE..d.n!%.L.....reele..l..y...	
LPUSP1	AEQRGVNQVEPLVYE-GGDVDDVIL----EQVIEFKP---DLLVTGADTEFPFSK-IAGAIGPRLARKAPISVIVVR	Size 153 % id 100
LVIS_1926	AEAFGEVHVQPIIAE-GGDVDDVIL----QKIVPDFQP---DLIVCGADVDFASHG-HPGAIGRLTKKADVSVIVIR	152 57
LSEI_2147	ATAFGVQNAQPIVAE-GRPAA-AIL----DEVIPAFKP---DLVIMGSHVR-RGRL-RLGHVASEVARDASVSVIIVR	153 44
LSA0042	AQEFQVQEVTPILGE-GNPGH-VIV----EEIIEFKP---DLVICGSKTK-PSKH-LIGTHASYLSKYAPSSVMVVR	156 42
PPYP_803599	AQDAGVQNVKCVTE-GDPGE-TIV----EEVIPAVQP---DLLVVGAESEKGLAR-HFGSQAAYMAKYAPITVT	140 39
LVIS0223	AHEAGVENVFPVIAE-GDPGE-EIV----NTVIPSIPK---EVLIVGSLAKKGPVK-YLGSQAAYMAKYAPISVLVVR	145 38
LVIS_1577	AERFGIQRVKVIGE-GDPGE-TIV----KKIIEVPK---DLLVVGSLSKSGVRK-YFGSQAAYMAKYSPISMIVR	150 37
LP_2877	ARDFGVTDVNAVDE-GDPGE-TIV----KTIIPALKP---DLLVIGSVAKHGVRK-YFGSQAAYMAKHAPISVLVIR	147 36
PPYP_803813	ALEFGIKKVQTYIAE-GEAGE-TIV----KNVIPSDVP---DLLVIGSSSKTGMAK-YFGSQAAYMAKHAPISVLVVR	149 35
OENOO_6501	AAKAGVKDIKEIVDE-GDAGE-AIV----KNVIPAVKA---DLLIIGSLSKKGISK-YFGSQAAYMAKYAPISVLIIR	148 34
LP_2993	AQAAGVTKVHAMIAE-GEAGE-TIV----KEVIPHVQP---DLLIIGSLAKKGIK-HFGSQAAYMAKYAPISVLVIR	143 33
MJ0577	EAKNMENIKKELEDVGVKVDIIVVGIPHEEIVKIAEDEGVDIIMGSHGKTNLKEILLGVSVTENVIKKSNKFPVLVVRKNS	162 16
ATP contacts:	<div style="display: inline-block; width: 100px; text-align: center;">R/P</div> <div style="display: inline-block; width: 100px; text-align: center;">R</div> <div style="display: inline-block; width: 100px; text-align: center;">Dim</div>	
Consensus	a...gv..!.....#.G.....Iv.....e!!p...p...Dl!.Gs..k.....Gs.a...ak.ap.svl!r...	

FIG. 1. Amino acid sequence alignment of Usp1 with homologous proteins from GenBank and MJ0577. Alignment was achieved by using online software (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>; version 5.4.1) (8). The protein origin and the GenBank codes are indicated (LP, *L. plantarum*; LVIS, *L. brevis*; LSEI, *L. casei*; LSA, *L. sakei*; PPYP, *P. pentosaceus*; OENOO, *O. oeni*; MJ, *M. jannaschii*). The consensus sequence of USP is also shown (capital letters correspond to conserved residues). Symbol comparison table: blosum62. Consensus symbols: !, anyone of IV; %, anyone of FY; #, anyone of NDQEBZ. The sizes and the global identity percentages between these sequences and Usp1 are indicated on the right. Residues of the ATP binding domain are boxed, and regions predicted to contact the adenosine (A), ribose (R), or triphosphate (P) are marked.

RESULTS

Nucleotide and protein sequence analysis of the *padA/padR-usp1* locus. To clone the complete LPNC8 sequence downstream of *usp1*, an inverse PCR amplification strategy was used (data not shown). This sequence was unknown at the time the present study was performed because the genome sequence of the *L. plantarum* WCFS1 strain had not yet been published. Analysis of the 462-bp sequence of *usp1* (AJ289188) revealed that the deduced product of 154 amino acid residues displayed 100% identity with the putative LP_3663 protein in the subsequent available genome of *L. plantarum* WCFS1 (<http://cmr.tigr.org/CMR/Search.shtml>) (19). LP_3663 is 1 of the 10 putative USPs of unknown function that are found in the WCFS1 strain. The paralogs that displayed the more significant identity with Usp1 in the WCFS1 strain are LP_2877 (36%), LP_2993 (33%), and LP_1163 (24%) (Fig. 1). Identity with the seven other *L. plantarum* putative USPs is below 20%. Multiple alignment of the USP amino acid sequences revealed that Usp1 displays higher identity with putative USPs of lactic acid bacteria belonging to the genus *Lactobacillus* (*L. brevis*, *L. sakei*, *L. casei*, and *L. salivarius*) but also with *Pedococcus* spp. and *Oenococcus oeni* (Fig. 1). The alignment revealed that these proteins can effectively be classified as members of the USP family (Pfam PF00582 or COG0589). They possess the

ATP binding domain (G-2X-G-9X-G-S/T) carried by the *Methanococcus jannaschii* MJ0577 Usp (36), but in a weakly conserved form. It is interesting that all of these species share many of the natural habitats of *L. plantarum* and that *P. pentosaceus*, the other lactic acid bacterium in which the PASR was studied, possesses two high similar Usp1 homologues (PPYP_803599 and PPYP_803813). Phylogenetic analysis of USPs primary sequences shows that PPYP_803599, PPYP_803813, LP2993, and Usp1 form a distinct subfamily (27) (Fig. 2).

Transcriptional analysis of *padA*, *padR*, and *usp1* in *L. plantarum*. Sequence analysis (<http://www.softberry.com>) of the *padR-usp1* intergenic region (Fig. 3A) did not detect the presence of a typical transcription terminator for *padR*. Moreover, our attempts to identify a putative *usp1* initiation transcription site by primer extension were unsuccessful, both with RNA extract from noninduced and *p*-coumaric-acid induced bacteria (data not shown). For Usp1, a strong transcription terminator ($\Delta G = -27 \text{ kcal mol}^{-1}$) was found 46 nucleotides downstream of the *usp1* stop codon. Downstream of this terminator we found the promoter of the *adhE* gene, which encodes a putative bifunctional alcohol and acetaldehyde dehydrogenase, a gene also found in the same locus in the strain WCFS1 (lp_3662). Northern blot hybridization was performed using RNA samples from cultures of *L. plantarum*, with or without

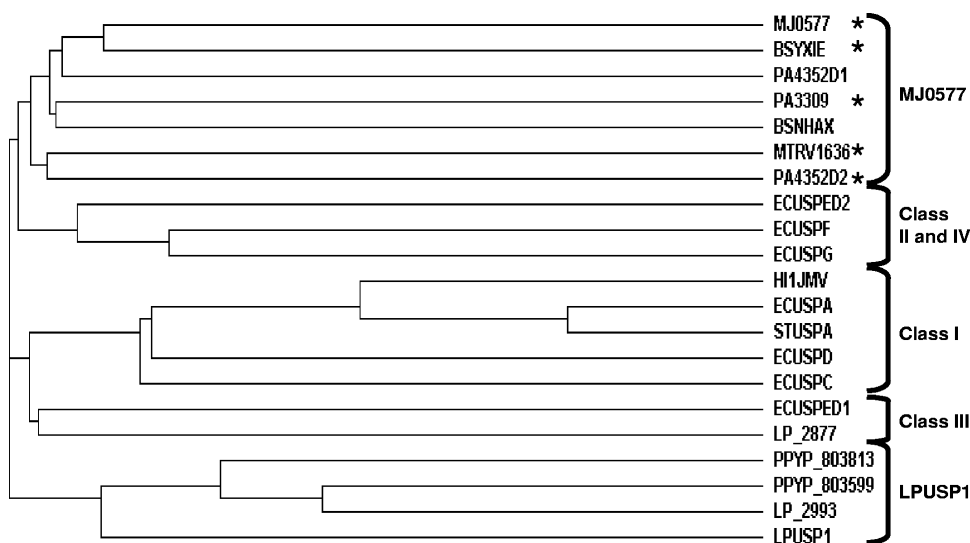


FIG. 2. Phylogenetic tree of USP. USPs are classified according to the method of Nachin et al. (27). Bacterium species and protein code in GenBank are indicated (MJ, *M. jannaschii*; BS, *B. subtilis*; PA, *Pseudomonas aeruginosa*; MT, *Mycobacterium tuberculosis*; EC, *E. coli*; HI, *Haemophilus influenzae*; ST, *Salmonella enterica* serovar Typhimurium; LP, *L. plantarum*; PP, *P. pentosaceus*). Proteins carrying the conserved MJ0577 ATP-binding domain are marked with an asterisk. This tree was produced using CLUSTAL W software (22a).

induction with *p*-coumaric acid as templates, and the *padR* and the *usp1* DNA probes (Fig. 3E). Two transcripts were observed with the two probes. The two bands of about 700 and 600 bases correspond to the size of the single transcript of *padR* and *usp1*, respectively. The 1,300-base band, which appears less intense than the 700- and 600-base bands, corresponds to the size of the *padR-usp1* cotranscript.

Since no *usp1* promoter and no *padR* transcription terminator could be identified in the intergenic region, the single transcripts could result from cleavage of the cotranscript. These results are in agreement with those previously reported using quantitative reverse transcription-PCR (RT-PCR), where after addition of *p*-coumaric acid, the *padR* and *usp1* relative transcript levels (RTLs) followed the same kinetics of expression as that of *padA*, but with lower folds of induction (37 and 13, respectively) compared to about 8,000 for *padA* (24). Unlike *usp1*, the expression of the three most similar paralogous genes in *L. plantarum* WCFS1 measured by quantitative RT-PCR in similar conditions was not induced by *p*-coumaric acid (data not shown), suggesting that these USPs have different functions in this bacterium.

Knockout of *usp1* and phenotypic characterization of the mutant strain. To study the putative role of Usp1 in the PASR, *usp1* was interrupted by deletion of an internal region corresponding to the putative ATP binding motif (for details, see Materials and Methods and Fig. 1 and 3A). Among the few putative mutants obtained after the Campbell double recombination event, the mutant, named LPNC8ΔUSP1 (Δ*usp1*), gave the expected amplicon size profile with selected primers (Fig. 3B) and was retained for further study. Southern blot with a DNA probe corresponding to the deleted fragment of *usp1* confirmed the presence of a deletion in the *usp1* sequence (Fig. 3C). A Northern blot analysis carried out with the same probe on RNA extracts from noninduced and *p*-coumaric acid-induced Δ*usp1* cultures gave no *usp1* transcript (Fig. 3E), whereas the result with the *padR* probe indicated that the

deletion of *usp1* did not affect *padR* expression, a condition essential to study the effect of the *usp1* deletion on PadR. In the Δ*usp1* mutant, the smaller size of the *padR-usp1* cotranscript of ~950 bases results from the 350-bp deletion in the *usp1* gene. Cultures of the wild-type or the Δ*usp1* mutant strain were induced at an optical density at 600 nm of 0.6 by the addition of 1.2 mM *p*-coumaric acid, and the PAD activity was tested in corresponding cell extracts (Fig. 3F). No significant difference in PAD activity was observed between the two strains, indicating that Usp1 was not absolutely required for PadR inactivation by *p*-coumaric acid.

The ability of the Δ*usp1* mutant to survive after exposure to different stresses was compared to that of the wild-type and the Δ*padR* mutant strain, in which Usp1 was overexpressed as previously demonstrated (24). These strains markedly differed in their ability to survive acid stress shock at pH 2.5 applied for 1 h at the onset of stationary growth phase (Fig. 3F). In these conditions, viability of the Δ*usp1* mutant strain was dramatically reduced and about threefold increased in the Δ*padR* strain when Usp1 was constitutively expressed. These results indicate that Usp1 is involved in the global acid stress response in this bacterium.

Expression of the *padA* locus genes in *E. coli*. The USP family has never been characterized in *L. plantarum* or in other lactic acid bacteria. Therefore, we introduced a functional *padA* locus in *E. coli*, which is devoid of the PASR genes, to avoid any possible interference with other genes from the host *L. plantarum* and to explore the possible interaction between the coexpressed proteins Usp1 and PadR. The complete DNA locus encompassing the *padA/padR-usp1* genes was PCR amplified and cloned into the vector TOPO PCR 2.1 to obtain the recombinant *E. coli* strain pLOCPAD (Fig. 4). Crude extracts from *E. coli* pLOCPAD, in the presence or absence of *p*-coumaric acid, exhibited a constitutive PAD activity of about 6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, a level similar to that of the *E. coli* pJPDC1 clone isolated from an *L. plantarum* genomic library in which

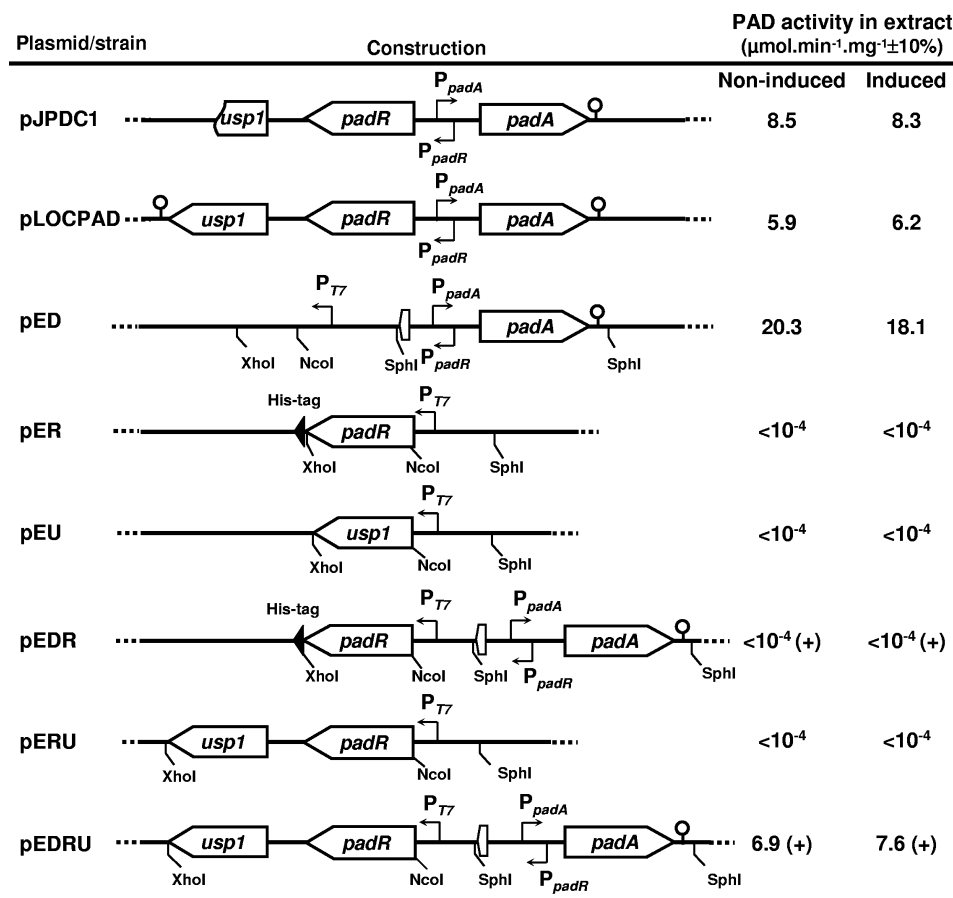


FIG. 4. Physical map of the different plasmid constructs of *padA*, *padR*, and *usp1* and the resulting PAD activity obtained from the host strains. Plasmids pJPDC1 and pLOCPAD were constructed with the pJDC9 and the TOPO PCR 2.1 vectors, respectively. Plasmids pED, pER, pEU1, pEDR, pERU, and pEDRU were constructed by using the pET28a+ vector. Closed triangles represent His tag fusions. Promoters are indicated by small arrows at the -35 position. Restriction sites used for construction are indicated. For PAD activity, I and NI correspond to protein extract from cells either induced (I) or not induced (NI) with 1.2 mM *p*-coumaric acid. (+), Induction with IPTG.

ple, a smear of mRNA of lower molecular mass may represent degradative products of the 1,300-base transcript. The cotranscription of *padR* and *usp1* in *E. coli* was confirmed by RT-PCR amplification experiments with mRNA prepared from *E. coli* pERU cells under inducing or noninducing conditions (1 mM IPTG) and the primers LPD5 and LPDU1 (Fig. 3A and D).

Expression of *usp1* in *E. coli* inactivates the PadR repressor.

To investigate the involvement of *usp1* in the PASR, *padR* and *usp1* genes were cloned individually or together in the pET28a+ vector under the control of the T7 promoter. In addition, *padA* was cloned under its own promoter into the SphI site of the pERU plasmid to serve as a reporter gene (Fig. 4). Control *E. coli* BL21 pER and *E. coli* BL21 pERU strains without *padA* did not display PAD activity. In *E. coli* BL21, the T7 promoter was operational to express the operon *padR-usp1*. SDS-PAGE analysis of crude extracts from *E. coli* BL21 pEDR and *E. coli* BL21 pEDRU showed that PadR was either produced at a low level without induction (data not shown) or overproduced with IPTG induction (Fig. 5D) and that Usp1 was well produced even without IPTG induction (Fig. 5D). This is in agreement with bioinformatics

analysis, which revealed that the *padR-usp1* intergenic region could serve as promoter in *E. coli*. No PAD activity was detected in either noninduced or *p*-coumaric acid-induced extracts from *E. coli* pEDR cells (Fig. 4). In contrast, crude extracts of *E. coli* BL21 pEDRU, in which PadR and Usp1 were produced at nearly the same concentrations (Fig. 5D), exhibited a constitutive PAD activity of $\sim 7 \mu\text{mol min}^{-1} \text{mg}^{-1}$ either with or without induction with *p*-coumaric acid (Fig. 4). This heterologous expression of the three genes of the *L. plantarum padA* locus in *E. coli* demonstrates that Usp1 is able to inactivate the PadR repressor when it is produced at near the same level in the bacteria.

Usp1 abolishes the binding of PadR with the *padA* promoter.

Mobility shift assays were carried out to study the influence of Usp1 on the ability of PadR to bind to the *padA* promoter. The PadR-His₆ and Usp1 proteins were overproduced in pER and pEU recombinant *E. coli* strains, respectively (Fig. 4 and Fig. 6A) and used in different EMSAs involving different combinations or treatments and with the appropriate controls (Fig. 6). Before carrying out EMSA experiments, the capacity of these two proteins to dimerize was tested by glutaraldehyde cross-linking experiments (Fig.

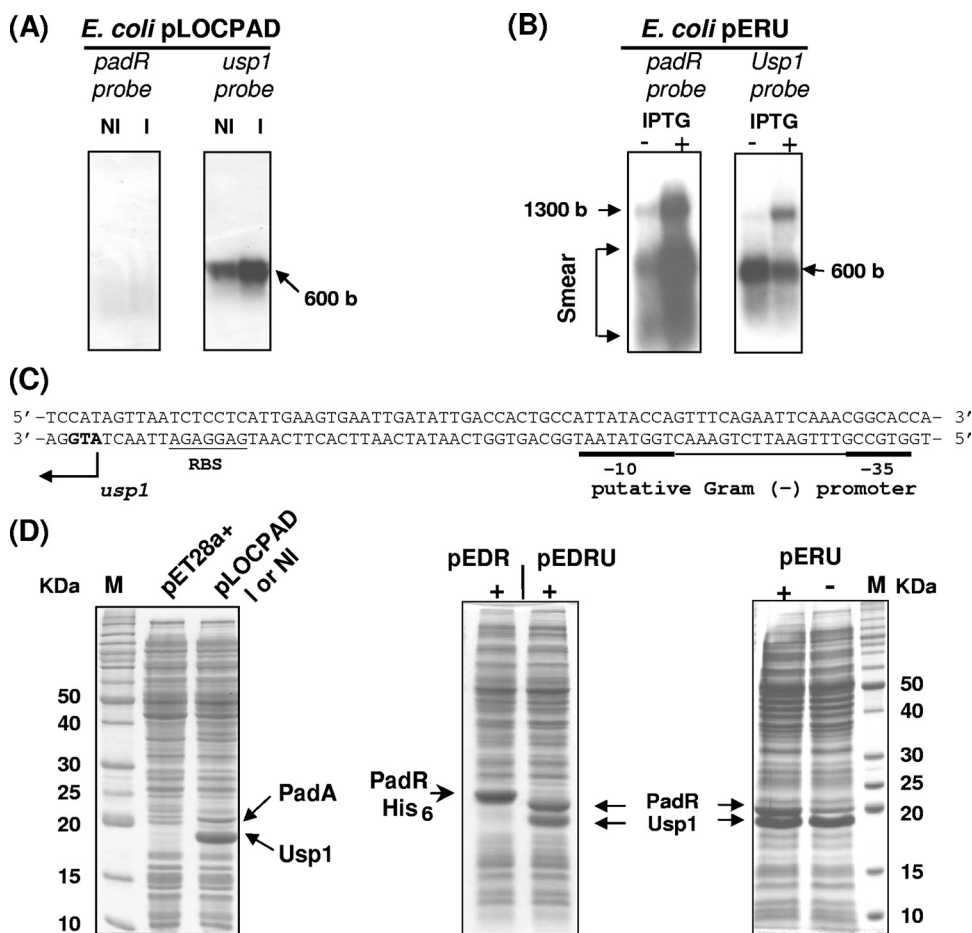


FIG. 5. Transcriptional analysis of *padR* and *usp1* in recombinant *E. coli* strains and corresponding SDS-PAGE of protein extracts. (A) Northern blot with RNA extract from noninduced (NI) and 1.2 mM *p*-coumaric acid 10-min-induced (I) cells. (B) Northern blot with RNA extract from 1 mM IPTG-induced (+) or not induced (−) cells. The 600- and 1,300-base bands correspond to the *usp1* and *padR-usp1* transcripts, respectively, while “Smear” probably represents degradative products from the 1,300-base transcript. (C) DNA sequence upstream of *usp1* indicating the −35 and −10 boxes that could serve as a promoter for *usp1* in the recombinant *E. coli* strains pLOCPAD and pERU. (D) SDS-PAGE analysis of crude protein extracts from the recombinant *E. coli* strains. pET28a+, *E. coli* TG1 with the vector pET28a+; I, 1.2 mM *p*-coumaric acid-induced cell extracts; − and +, noninduced (−) and 1 mM IPTG-induced (+) cell extract. M, molecular mass standards.

6B). Although PadR forms dimers and oligomers at very low concentrations of glutaraldehyde, Usp1 did not, despite its amino acid sequence showing a putative pattern of dimerization (Fig. 1) and despite the high concentrations of glutaraldehyde tested. EMSA results indicate that PadR was able to bind specifically to the *padA* promoter and, depending on its concentration, to form one or two complexes with the *padA* promoter probe (Fig. 6C). This binding was specific, as demonstrated by the disappearance of the shift when PadR was preincubated in excess of unlabeled specific *padA* promoter and by the conservation of the shift in the presence of high concentrations of the unspecific competitor poly(dI-dC) (Fig. 6D). Preincubation of PadR with 10 mM concentration of *p*-coumaric, ferulic, and *o*-coumaric acid was unable to abolish the probe shift (Fig. 6E).

Taking into account the results of our recent work on the PASR in *B. subtilis* (33), which suggested a possible interference of MgCl₂ in EMSA, the EMSA was performed in the same conditions as those shown in Fig. 6E but without MgCl₂ in the binding buffer (Fig. 6F). In contrast to the results ob-

tained with *B. subtilis*, the addition of phenolic acid did not abolish the binding of PadR to the *padA* promoter. To investigate whether Usp1 binds to the *padA* promoter, serving as a competitor for this promoter or modifying the affinity of PadR to the *padA* promoter, different EMSAs with the *padA* promoter were carried out (Fig. 6G and H). A concentrate of crude protein extract from *E. coli* carrying the vector pET28a+ without the *usp1* gene was unable to abolish the binding of the *padA* promoter with PadR (Fig. 6G). Usp1 was also unable to bind to the *padA* promoter (Fig. 6H), demonstrating that Usp1 had no affinity for the *padA* promoter and is not a competitor of *padR* for the *padA* promoter.

Since Usp1 inactivates PadR (Fig. 4), EMSAs were carried out with PadR preincubated or not with Usp1 extract, in the same range of concentrations (Fig. 6I). The results indicate that Usp1 abolishes the binding of PadR to the *padA* promoter, and they support the inactivation of PadR by Usp1 in *E. coli* pEDRU (Fig. 4), despite high levels of PadR in the bacteria (Fig. 5D). Since PadR and Usp1 might form a coprecipitate, a nonspecific interaction that could account for the inability of PadR to bind to the

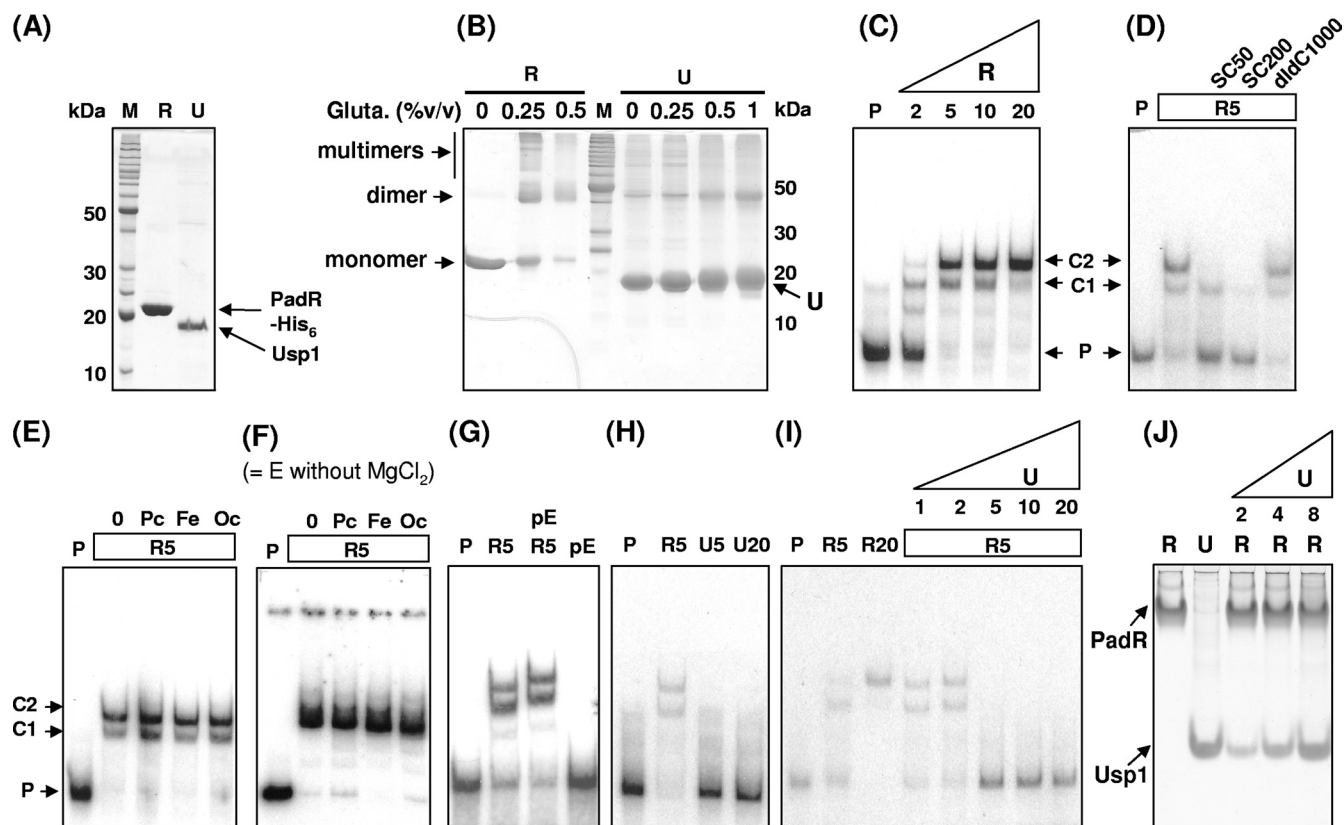


FIG. 6. EMSA analyses of PadR and Usp1 with the *padA* promoter. (A) SDS-PAGE of proteins extracts containing purified PadR and overexpressed Usp1 used in the EMSA. M, molecular mass standard; R, 2.5 μ g of purified PadR-His₆; U, 2 μ g of protein extract from *E. coli* pEU in which Usp1 represents ca. 90% of the total protein in the extract. (B) SDS-PAGE of PadR and Usp1 preincubated 10 min with increasing concentrations of glutaraldehyde. (C) EMSA of increasing concentrations from 2 to 20 nM PadR (R) with the *padA* promoter probe. P, *padA* promoter DNA probe without PadR. C1 and C2 indicate putative complexes formed between the DNA probe and PadR. (D) EMSA with probe and 5 nM PadR (R5) with addition of a 50-fold (SC50) or a 200-fold (SC200) excess of unlabeled *padA* promoter fragment as a specific competitor or a 1,000-fold (dIdC1000) excess of competitor poly(dI-dC). (E) EMSA with probe and 5 nM PadR (R5) preincubated for 10 min with 10 mM *p*-coumaric (Pc), ferulic (Fe), and *o*-coumaric (Oc) acid. (F) Same as panel E, but without MgCl₂ in the binding buffer. (G) EMSA with probe and 5 nM PadR without (R5) and with (R5 pE) total protein extract from *E. coli* pET28a+ at a protein concentration equivalent to 10 nM Usp1. pE corresponds to the EMSA with probe and the extract of *E. coli* pET28a+ without cloned gene. (H) EMSA with probe and 5 (U5) or 20 (U20) nM Usp1 extract. R5, the EMSA with probe and 5 nM PadR was used as the positive binding control. (I) EMSA with probe and 5 nM (R5) and 20 nM (R20) PadR, followed by EMSA with probe and 5 nM (R5) PadR, in which PadR was preincubated before the binding reaction for 10 min with increasing concentrations of Usp1 at 1, 2, 5, 10, and 20 nM. (J) Nondenaturing PAGE of PadR and Usp1 produced from recombinant *E. coli* strains pER and pEU, respectively. These extracts were similar to extracts R and U analyzed in SDS-PAGE in the panel A. R, 3 μ g of PadR; U, 4 μ g of pEU extract. The lanes correspond to mixes of 4 μ g of PadR incubated for 10 min with increasing amounts of pEU extract (2, 4, and 8 μ g) in 20 μ l of binding buffer. Binding reactions for panels C, D, E, G, H, and I were carried out in standard conditions (with 2.5 mM MgCl₂).

padA promoter, individual and mixed protein extracts of the two proteins were resolved in nondenaturing PAGE (Fig. 6J) without heating the sample before loading. Although the two proteins have similar migration rates in SDS-PAGE (Fig. 6A), they display distinct migration rates in their native form in a PAGE (Fig. 6J, lanes R and U). Therefore, mixed extracts from purified PadR and increasing concentrations of Usp1 extract were incubated and resolved by PAGE (Fig. 6J, lanes U2R to U8R). In the mixed extracts, each protein conserved perfectly the same migration rate and no other protein band corresponding to a putative PadR-Usp1 coprecipitate was detected.

DISCUSSION

The aim of this study was to elucidate genetic and biochemical mechanisms of the phenolic acid stress response in *L.*

plantarum, a specific and strong stress response induced by some of these chemicals. Sequence and transcriptional analysis of the *L. plantarum padA* locus indicates that *padR*, which encodes the negative repressor of *padA*, is divergently oriented from *padA*. PadR also forms a bicistronic operon with a gene of unknown function named *usp1*, since its deduced product shares significant identity with proteins belonging to the USP family. However, the phylogenetic tree (Fig. 2) clearly indicates that Usp1 forms a subfamily with another putative USP of *L. plantarum* (LP-2993) and two putative USPs from *P. pentosaceus*. The expression of the *padR-usp1* operon specifically induced by phenolic acids is autoregulated by PadR itself (24). Taking together the fact that *usp1* is overexpressed during the PASR and the fact that expression of USPs is induced by a large variety of stresses such as the entry in the stationary growth phase and various situations of starvation of bacteria

(29, 34), we hypothesized that Usp1 may be involved in the PASR. To address this, a $\Delta usp1$ mutant was constructed and tested for its ability to induce the *p*-coumaric acid-dependent PAD activity. Although the $\Delta usp1$ mutant conserved the wild-type phenotype, the *usp1* expression was specifically induced by *p*-coumaric acid, whereas the three *usp1* highest similar paralogous genes of *L. plantarum* were not. These results prompted us to study the possible interaction between PadR and Usp1 in a heterologous system devoid of the PASR.

Coexpression of *padR* and *usp1* demonstrated that Usp1 produced in *E. coli* was able to inactivate PadR, while the addition of *p*-coumaric acid to a recombinant *E. coli* pEDR expressing PadR could not, as it was also observed in recombinant *E. coli* pJPADP1 expressing the *padAR* operon from *P. pentosaceus* (4). All of these results provide evidence that PadR is not inactivated directly by *p*-coumaric acid or by one of the six putative USPs found in the *E. coli* genome (28) and independently of the stage of growth (data not shown). This is in agreement with the phylogenetic sequence analysis, which clusters Usp1 in a distinct family from the *E. coli* USPs (Fig. 2).

The capacity of Usp1 to inactivate PadR was confirmed by EMSA with PadR preincubated or not with Usp1. It is to our knowledge the first time that the capacity of a USP to inactivate a transcriptional regulator is demonstrated, and it is possible that Usp1 modulates other regulators in the same fashion. USPs could act as coordinators of regulator status during stresses. Although PadR and Usp1 possess conserved and characterized dimerization domains (16, 27) (Fig. 1), Usp1 does not seem to be able to form homodimers nor heterodimers with PadR. According to the putative ATP binding domains of PadR (16) and Usp1 (Fig. 1) (27), ATP exchange between Usp1 and PadR could be suspected to be involved in the inactivation of PadR. Usp1 should not act directly with PadR in living *L. plantarum* cells, but could act as an ATP donor. It was found in *E. coli* that the addition of ATP to GroEL complexes releases the UspG form, and conversely, that the presence of ATP blocks the interaction of UspG with GroEL (5). Moreover, it was demonstrated in *E. coli* that the MJ0577 protein, a Usp1 paralogue able to bind ATP, requires an additional cellular factor for ATP hydrolysis. Phosphorylation studies of PadR and Usp1, and the possible transfer of phosphate from Usp1 to PadR during the PASR, will be studied soon. Such a mechanism might depend on the environment of the regulators. Hence, to address this, it is necessary to overexpress and purify these two proteins from the native host *L. plantarum*, as was done for LmrR (26). Functional analysis of the proteins expressed in *E. coli* versus those expressed in its native host *L. plantarum* will be undertaken.

In mammals, phenolic acids, particularly ferulic and caffeic acids, are diet compounds strongly associated with a reduced risk of developing chronic diseases (25). This effect is in part attributed to their antioxidant properties. However, studies have shown that caffeic acid can also trigger DNA degradation in the presence of copper ions due to its pro-oxidant activity (35, 37). Caffeic acid, a PASR inducer like ferulic and *p*-coumaric acids, has a strong pro-oxidant effect due to the presence of an ortho-dihydroxyl group that chelates O_2 with Cu^{2+} ions and generates reactive oxygen species, which are responsible for the DNA damage. It is important to note that in *E. coli*, UspA is required for resistance to DNA-damaging

agents (11, 17). Thus, we could hypothesize that Usp1, a UspA family protein, might also be involved in resistance to DNA-damaging agents in *L. plantarum*, and it may explain the advantage for this bacterium to have its expression induced by phenolic acids, potentially DNA-damaging agents. Nevertheless, this hypothesis does not exclude that Usp1 might be directly involved in the PASR. The lack of an evident phenotype of the *usp1* mutant in the PASR could be explained by the presence of another protein with functional redundancy expressed when *usp1* is knocked out. We provided evidence for this kind of relay in a recent analysis of the *B. subtilis* response to salicylic acid, another phenolic acid (13). We showed that the absence of sensitivity to salicylic acid of a mutant in the *bsdBCD* operon defective in the BsdB, C, and D enzymes involved in the decarboxylation of phenylacrylic and hydroxybenzoic acids was taken over by the expression of the *padC* gene involved in the PASR. In contrast to the simple mutant *bsdBCD*, the double mutant *bsdBCD-padC* was sensitive to this phenolic acid. Recent analyses of USPs function in several groups of microorganisms highlight the complexity of the mechanism of action of proteins suspected to be components of the stress response network in cells, but their role remains most often enigmatic (22). Although we provide evidence that *usp1* is involved in the global acid stress response of cells entering into the stationary phase, a comparison of transcriptomic and proteomic data from the *usp1* mutant and *padR* mutants versus the wild-type strain will be undertaken to obtain an overview of the Usp1 and PadR roles in the behavior of *L. plantarum*.

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REFERENCES

1. Aukrust, T., and I. F. Nes. 1988. Transformation of *Lactobacillus plantarum* with the plasmid pTV1 by electroporation. FEMS Microbiol. Lett. **52**:127–132.
2. Axelsson, L. 1996. Proceedings of the Fifth Symposium on Lactic Acid Bacteria: genetics, metabolism, and applications, Veldhoven, The Netherlands. Antonie van Leeuwenhoek **70**(2–4):97–358.
3. Barthelmebs, L., C. Diviès, and J.-F. Cavin. 2000. Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Appl. Environ. Microbiol. **66**:3368–3375.
4. Barthelmebs, L., B. Lecomte, C. Diviès, and J.-F. Cavin. 2000. Inducible metabolism of phenolic acids in *Pediococcus pentosaceus* is encoded by an autoregulated operon which involves a new class of negative transcriptional regulator. J. Bacteriol. **182**:6724–6731.
5. Bochkareva, E. S., A. S. Girshovich, and E. Bibi. 2002. Identification and characterization of the *Escherichia coli* stress protein UP12, a putative in vivo substrate of GroEL. Eur. J. Biochem. **269**:3032–3040.
6. Cavin, J.-F., L. Barthelmebs, and C. Diviès. 1997. Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, overexpression in *Escherichia coli*, purification, and characterization. Appl. Environ. Microbiol. **63**:1939–1944.
7. Cohen, S. N., A. C. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
8. Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**:10881–10890.
9. De Man, P. J., M. Rogosa, and M. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130–135.

10. Derré, I., G. Rapoport, and T. Msadek. 1999. CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in gram-positive bacteria. *Mol. Microbiol.* **31**:117–131.
11. Diez, A., N. Gustavsson, and T. Nyström. 2000. The universal stress protein A of *Escherichia coli* is required for resistance to DNA damaging agents and is regulated by a RecA/FtsK-dependent regulatory pathway. *Mol. Microbiol.* **36**:1494–1503.
12. Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High-efficiency transformation of *Escherichia coli* by high-voltage electroporation. *Nucleic Acids Res.* **16**:6127–6145.
13. Duy, N. V., U. Mader, N. P. Tran, J.-F. Cavin, T. Tam le, D. Albrecht, M. Hecker, and H. Antelmann. 2007. The proteome and transcriptome analysis of *Bacillus subtilis* in response to salicylic acid. *Proteomics* **7**:698–710.
14. Ferguson, L. R., I. F. Lim, A. E. Pearson, J. Ralph, and P. J. Harris. 2003. Bacterial antimutagenesis by hydroxycinnamic acids from plant cell walls. *Mutat. Res.* **542**:49–58.
15. Gibson, T. J. 1984. Studies on the Epstein-Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge, United Kingdom.
16. Gury, J., L. Barthelmebs, N. P. Tran, C. Diviès, and J.-F. Cavin. 2004. Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding *padA* gene of *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* **70**:2146–2153.
17. Gustavsson, N., A. Diez, and T. Nyström. 2002. The universal stress protein paralogs of *Escherichia coli* are co-ordinately regulated and co-operate in the defense against DNA damage. *Mol. Microbiol.* **43**:107–117.
18. Hols, P., T. Ferain, D. Garmyn, N. Bernard, and J. Delcour. 1994. Use of homologous expression-secretion signals and vector-free stable chromosomal integration in engineering of *Lactobacillus plantarum* for alpha-amylase and levanase expression. *Appl. Environ. Microbiol.* **60**:1401–1413.
19. Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* **100**:1990–1995.
20. Kovacikova, G., W. Lin, and K. Skorupski. 2005. Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in *Vibrio cholerae*. *Mol. Microbiol.* **57**:420–433.
21. Kovacikova, G., W. Lin, and K. Skorupski. 2003. The virulence activator AphA links quorum sensing to pathogenesis and physiology in *Vibrio cholerae* by repressing the expression of a penicillin amidase gene on the small chromosome. *J. Bacteriol.* **185**:4825–4836.
22. Kvint, K., L. Nachin, A. Diez, and T. Nyström. 2003. The bacterial universal stress protein: function and regulation. *Curr. Opin. Microbiol.* **6**:140–145.
- 22a. Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947–2948.
23. Li, Y., Q. Peng, D. Selimi, Q. Wang, A. O. Charkowski, X. Chen, and C. H. Yang. 2009. The plant phenolic compound *p*-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system. *Appl. Environ. Microbiol.* **75**:1223–1228.
24. Licandro-Seraut, H., J. Gury, N. P. Tran, L. Barthelmebs, and J.-F. Cavin. 2008. Kinetics and intensity of the expression of genes involved in the stress response tightly induced by phenolic acids in *Lactobacillus plantarum*. *J. Mol. Microbiol. Biotechnol.* **14**:41–47.
25. Liu, R. H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* **134**:3479S–3485S.
26. Madoori, P. K., H. Agustindari, A. J. Driessen, and A. M. Thunnissen. 2009. Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition. *EMBO J.* **28**:156–166.
27. Nachin, L., L. Brive, K. C. Persson, P. Svensson, and T. Nyström. 2008. Heterodimer formation within universal stress protein classes revealed by an in silico and experimental approach. *J. Mol. Biol.* **380**:340–350.
28. Nachin, L., U. Nannmark, and T. Nyström. 2005. Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and mobility. *J. Bacteriol.* **187**:6265–6272.
29. Nyström, T., and F. C. Neidhardt. 1992. Cloning, mapping, and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. *Mol. Microbiol.* **6**:3187–3198.
30. Posno, M., R. J. Leer, N. Van Luik, M. J. F. Van Giezen, P. T. H. M. Heuvelmans, B. C. Lockman, and P. H. Pouwels. 1991. Incompatibility of *Lactobacillus* vectors with replicons derived from small cryptic *Lactobacillus* plasmids and segregational instability of the introduced vectors. *Appl. Environ. Microbiol.* **57**:1822–1828.
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
32. Scalbert, A., and G. Williamson. 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**:2073S–2085S.
33. Tran, N. P., J. Gury, V. Dartois, T. K. Nguyen, H. Seraut, L. Barthelmebs, P. Gervais, and J.-F. Cavin. 2008. Phenolic acid-mediated regulation of the *padC* gene, encoding the phenolic acid decarboxylase of *Bacillus subtilis*. *J. Bacteriol.* **190**:3213–3224.
34. VanBogelen, R. A., M. E. Hutton, and F. C. Neidhardt. 1990. Gene-protein database of *Escherichia coli* K-12, edition 3. *Electrophoresis* **11**:1131–1166.
35. Yamanaka, N., O. Oda, and S. Nagao. 1997. Prooxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu²⁺-induced low density lipoprotein oxidation. *FEBS Lett.* **405**:186–190.
36. Zarebinski, T. I., L. W. Hung, H. J. Mueller-Dieckmann, K. K. Kim, H. Yokota, R. Kim, and S. H. Kim. 1998. Structure-based assignment of the biochemical function of a hypothetical protein: a test case of structural genomics. *Proc. Natl. Acad. Sci. USA* **95**:15189–15193.
37. Zheng, L. F., F. Dai, B. Zhou, L. Yang, and Z. L. Liu. 2008. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu(II) ions: mechanism and structure-activity relationship. *Food Chem. Toxicol.* **46**:149–156.